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Subunit compensation and plasticity of synaptic GABA_A receptors induced by ethanol in $\alpha 4$ subunit knockout mice

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There is considerable evidence that ethanol (EtOH) potentiates γ -aminobutyric acid type A receptor (GABA_AR) action, but only GABA_ARs containing δ subunits appear sensitive to low millimolar EtOH. The $\alpha 4$ and δ subunits co-assemble into GABA_ARs which are relatively highly expressed at extrasynaptic locations in the dentate gyrus where they mediate tonic inhibition. We previously demonstrated reversible- and time-dependent changes in GABA_AR function and subunit composition in rats after single-dose EtOH intoxication. We concluded that early tolerance to EtOH occurs by over-activation and subsequent internalization of EtOH-sensitive extrasynaptic $\alpha 4\beta\delta$ -GABA_ARs. Based on this hypothesis, any highly EtOH-sensitive GABA_ARs should be subject to internalization following exposure to suitably high EtOH doses. To test this, we studied the GABA_ARs in mice with a global deletion of the $\alpha 4$ subunit (KO). The dentate granule cells of these mice exhibited greatly reduced tonic currents and greatly reduced potentiation by acutely applied EtOH, whereas synaptic currents showed heightened sensitivity to low EtOH concentrations. The hippocampus of naïve KO mice showed reduced δ subunit protein levels, but increased $\alpha 2$, and $\gamma 2$ levels compared to wild-type (WT) controls, suggesting at least partial compensation by these subunits in synaptic, highly EtOH-sensitive GABA_ARs of KO mice. In WT mice, cross-linking and Western blot analysis at 1 h after an EtOH challenge (3.5 g/kg, i.p.) revealed increased intracellular fraction of the $\alpha 1$, $\alpha 4$, and δ , but not $\alpha 2$, $\alpha 5$, or $\gamma 2$ subunits. By contrast, we observed significant internalization of $\alpha 1$, $\alpha 2$, δ , and $\gamma 2$ subunits after a similar EtOH challenge in KO mice. Synaptic currents from naïve KO mice were more sensitive to potentiation by zolpidem (0.3 μ M, requiring $\alpha 1/\alpha 2$, inactive at $\alpha 4/5$ GABA_ARs) than those from naïve WT mice. At 1 h after EtOH, synaptic currents of WT mice were unchanged, whereas those of KO mice were significantly less sensitive to zolpidem, suggesting decreases in functional $\alpha 1/2\beta\gamma$ GABA_ARs. These data further support our hypothesis that EtOH intoxication induces GABA_AR plasticity via internalization of highly EtOH-sensitive GABA_ARs.

Keywords: tolerance, dependence, withdrawal, internalization, dentate gyrus, synaptic transmission, alcohol, receptor trafficking

INTRODUCTION

The mammalian γ -aminobutyric acid type A receptors (GABA_ARs) are heteropentameric chloride channel proteins formed from a family of 19 related subunits, named $\alpha 1$ – $\alpha 6$, $\beta 1$ – $\beta 3$, $\gamma 1$ – $\gamma 3$, δ , ϵ , θ , π , and $\rho 1$ – $\rho 3$ (Olsen and Sieghart, 2008). Synaptic GABA_ARs mediate the bulk of fast (phasic) inhibition, while extrasynaptic GABA_ARs provide a sustained (tonic) inhibitory influence on brain neurotransmission. GABA_ARs are important targets for drugs such as benzodiazepines (BZs), barbiturates, neurosteroids, ethanol (EtOH), other general anesthetics, and a number of picrotoxin-like convulsants. Differences in GABA_AR subunit composition are major determinants of channel kinetics, sensitivity to GABA, and cellular localization to synaptic and

extrasynaptic membranes (Olsen and Sieghart, 2009). For example, the presence of $\alpha 4$ and δ subunits in GABA_ARs results in their extrasynaptic localization (Nusser et al., 1998; Wei et al., 2003; Liang et al., 2006), sensitivity to low concentrations of ambient GABA (Wei et al., 2003; Chandra et al., 2006), insensitivity to the classical BZs such as diazepam, and responsiveness to low millimolar concentrations of EtOH (Sundstrom-Poromaa et al., 2002; Wallner et al., 2003; Wei et al., 2004; Liang et al., 2008). EtOH potentiation of extrasynaptic GABA_ARs is not universally accepted, several labs have reported a lack of EtOH potentiation of recombinant δ subunit-containing GABA_ARs (Borghese and Harris, 2007; Korpi et al., 2007). Also, at least some of EtOH's acute effects on GABAergic transmission are mediated

by enhancement of presynaptic GABA release (Roberto et al., 2006).

It is widely accepted that an important aspect of EtOH's acute action on the brain is enhancement of inhibition mediated by GABA_ARs, while withdrawal involves a reduction in GABA_AR-mediated inhibition (Kumar et al., 2009). A well-substantiated theory of how repeated use of EtOH leads to dependence is that the chronic repetition of the mini-withdrawals leads to a persistent state of withdrawal (alcohol withdrawal syndrome, AWS) in which the withdrawals are more severe and long-lasting, eventually permanent (Becker, 2008). Many of the behavioral and pharmacological changes associated with AWS and dependence may be explained by alterations in the function of GABA_ARs (Kumar et al., 2009). Chronic EtOH, particularly chronic intermittent EtOH (CIE) administration in rodents produces long-lasting alterations in GABA_AR subunit composition, localization, and function (Mhatre and Ticku, 1992; Devaud et al., 1997; Cagetti et al., 2003; Liang et al., 2006). Even a single intoxicating EtOH dose is capable of inducing such alterations, albeit fully reversible (Liang et al., 2007). However, the cellular and molecular mechanisms behind EtOH-induced GABA_AR plasticity are not well understood.

Here we studied the early events in the reversible GABA_AR plasticity produced by EtOH intoxication. We hypothesized that early tolerance to EtOH occurs by over-activation and subsequent internalization of EtOH-sensitive extrasynaptic $\alpha 4\beta\delta$ -GABA_ARs (Liang et al., 2007; Shen et al., 2011). Based on this hypothesis, any highly EtOH-sensitive GABA_ARs should be subject to internalization following exposure to moderate-high EtOH doses. To test this, we examined the biochemistry and pharmacology of GABA_ARs in mice with a global deletion of the $\alpha 4$ subunit (KO). We previously demonstrated that dentate granule cells (DGCs) of these mice exhibit greatly reduced tonic currents (Chandra et al., 2006; Liang et al., 2008) and greatly reduced potentiation by acutely applied EtOH (Liang et al., 2008). However, behavioral responses of KO mice to acute EtOH are almost unchanged (Chandra et al., 2008); this may be attributed to the heightened sensitivity of synaptic currents to low EtOH concentrations (Liang et al., 2008). Therefore, we examined the function, pharmacology and subunit composition of synaptic GABA_ARs before and after EtOH intoxication in KO mice and their wild-type (WT) counterparts. Our data suggest that genetic deletion of $\alpha 4$ subunits results in compensatory alterations in synaptic GABA_AR subunits which undergo rapid internalization following EtOH intoxication.

MATERIALS AND METHODS

MICE

The $\alpha 4$ KO and WT littermate control mice were produced from heterozygous breeding pairs on a C57BL/6J N7 genetic background. Details of mouse production and genotyping have been previously reported (Chandra et al., 2006). The Institutional Animal Care and Use Committee approved all animal experiments. Only adult male (3–9 months old) mice were used in the experiments described here. Animals of different age were distributed randomly across the different experiments and both biochemical and electrophysiological experiments were often conducted on different hippocampal slices from the same animal. In some

experiments, mice received an injection of pyrazole (68 mg/kg, i.p., Sigma, St Louis, MO, USA) 30 min prior to injection of saline or EtOH (3.5 g/kg, i.p.).

BRAIN SLICE PREPARATION AND WESTERN BLOTTING

Coronal slices (400 μ m thick) of mouse dorsal hippocampus were obtained using standard techniques (Spigelman et al., 2003). Briefly, mice were anesthetized with isoflurane, decapitated, and sections obtained using a vibrating blade microtome (VT1200S, Leica Microsystems, Bannockburn, IL, USA) while immersed in cold (0–4°C) artificial cerebrospinal fluid (ACSF) composed of (in mM): NaCl, 125; KCl, 2.5; CaCl₂, 2; MgCl₂, 2; NaHCO₃, 26 and D-glucose, 10. The ACSF was continuously bubbled with a 95/5% mixture of O₂/CO₂ to ensure adequate oxygenation of slices and a pH of 7.4. The dentate gyrus and CA1 regions were microdissected from individual sections, and incubated in a small volume chamber with or without the protein cross-linking reagent, bis(sulfosuccinimidyl)suberate (BS³) in ACSF at 4°C according to (Grosshans et al., 2002). BS³ is bifunctional and cross-links all proteins exposed to the medium, i.e., cell-surface proteins. These large complexes do not enter the gel and are retained at the top; thus the band of protein at the identified M_r corresponds to that fraction that is intracellular only. The difference between that value and the amount from an equivalent adjacent slice, untreated, and thus total, represents the surface pool. After incubation of brain sections in the absence (total) or presence (intracellular) of BS³, sections were washed three-times with Tris wash buffer (pH 7.6) and homogenized in a buffer composed of 1% SDS, 1 mM EDTA, and 10 mM Tris, pH 8.0. Protein aliquots (40 μ g) from samples were separated on 10% SDS-polyacrylamide gel electrophoresis under reducing conditions using the BioRad Mini-Protean 3 Cell system. Proteins were transferred to PVDF membranes (Immun-Blot PVDF membrane, 0.2 mm, BioRad) by wet transfer (BioRad, Hercules, CA, USA). Blots were probed with anti-peptide $\alpha 1$ (proprietary N-terminus sequence), $\alpha 2$ (322–357), $\alpha 4$ (379–421), $\alpha 5$ (337–388), $\gamma 1$ (1–39), $\gamma 2$ (319–366), or δ (1–44) antibodies (all at 1 mg/ml), followed by HRP-conjugated anti-rabbit secondary antibody (1:5000 dilution) and bands detected by ECL detection kit (Amersham Pharmacia UK), apposed to X-ray film under non-saturating conditions. The $\alpha 1$ GABA_AR antibody was obtained from Novus Biologicals (Littleton, CO, USA). β -actin (Sigma, St. Louis, MO, USA) or GAPDH (Santa Cruz Biotech, Santa Cruz, CA, USA) antibodies were used as a loading controls. Bands from different samples corresponding to the appropriate subunit were analyzed and absorbance values compared by densitometry using ImageJ (NIH, Bethesda, MD, USA). Group differences were evaluated by unpaired *t*-test. *p* < 0.05 was considered statistically significant.

ELECTROPHYSIOLOGICAL RECORDINGS

Whole-cell patch clamp recordings were obtained from cells located in the DGC layers at 34 ± 0.5°C during perfusion with ACSF using patch pipettes containing (in mM): cesium gluconate, 135; MgCl₂, 2; CaCl₂, 1; ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid, 11; N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, 10; K₂ATP, 2; Na₂GTP, 0.2; pH adjusted to 7.25 with CsOH. GABA_AR-mediated

mIPSCs were pharmacologically isolated by adding tetrodotoxin (TTX, 0.5 μ M), D(-)-2-amino-5-phosphonopentanoate (APV, 40 μ M), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10 μ M), and CGP 54626 (1 μ M) to the ACSF from stock solutions. Stock solutions of CNQX were made with pure dimethyl sulfoxide (DMSO). Final concentration of DMSO did not exceed 42 μ M in the recording chamber. Signals were recorded in voltage-clamp mode with an amplifier (Axoclamp-2B, Molecular Devices, Sunnyvale, CA, USA). Whole-cell access resistances were in the range of 8–20 M Ω before electrical compensation by \sim 70%. During voltage-clamp recordings, access resistance was monitored by measuring the size of the capacitive transient in response to a 5-mV step command, and experiments were abandoned if changes $>$ 20% were encountered. At least 10 min was allowed for equilibration of the pipette solution with the intracellular milieu before commencing recordings. Data were acquired with pClamp 9 software (Molecular Devices), digitized at 20 kHz (Digidata 1200B, Molecular Devices) and analyzed using the Clampfit software (Molecular Devices) and the Mini Analysis Program (versions 5.2.2 and 5.4.8, Synaptosoft).

DETECTION AND ANALYSIS OF mIPSCs

The recordings were low-pass filtered off-line (Clampfit software) at 2 kHz. The mIPSCs were detected with threshold criteria of: 5 pA, amplitude and 20 fC, charge transfer. Frequency of mIPSCs was determined from all automatically detected events in a given 100 s recording period. For kinetic analysis, only single event mIPSCs with a stable baseline, sharp rising phase, and exponential decay were chosen during visual inspection of the recording trace. Double and multiple peak mIPSCs were excluded. The mIPSC kinetics were obtained from analysis of the averaged chosen single events ($>$ 120 events/100 s recording period) aligned with half rise time in each cell. Decay time constants were obtained by fitting a double exponential to the falling phase of the averaged mIPSCs. The investigator performing the recordings and mIPSC analysis was blind to the mouse genotypes.

RESULTS

COMPENSATORY CHANGES IN GABA_AR SUBUNIT LEVELS IN KO MICE

We first studied compensatory changes in GABA_AR subunit levels in the microdissected DG/CA1 areas of hippocampal slices from untreated α 4 KO mice compared to their WT counterparts. The DG/CA1 area was kept intact to maximize tissue yield for the protein assays and because previous studies demonstrated similar EtOH-induced alterations in the two hippocampal regions (Liang et al., 2007). As illustrated in **Figure 1**, KO mice showed significant decreases in both α 4 and δ subunit levels ($p < 0.05$). By contrast, significant increases were observed in the α 2 ($p = 0.032$) and γ 2 ($p = 0.007$) subunit levels. Although increases in γ 2 subunits confirmed previous results (Liang et al., 2008), the compensatory increases in α 2 subunits were surprising given the normally limited abundance of the predominantly extrasynaptic α 4 and δ subunits in WT mice. There was considerable variability in the γ 1 subunit expression and the apparent increases did not reach statistical significance ($p = 0.28$). Although compensatory increases in α 5 subunit were expected given its relatively high abundance in the extrasynaptic GABA_ARs of the CA1 region (Caraiscos et al., 2004),

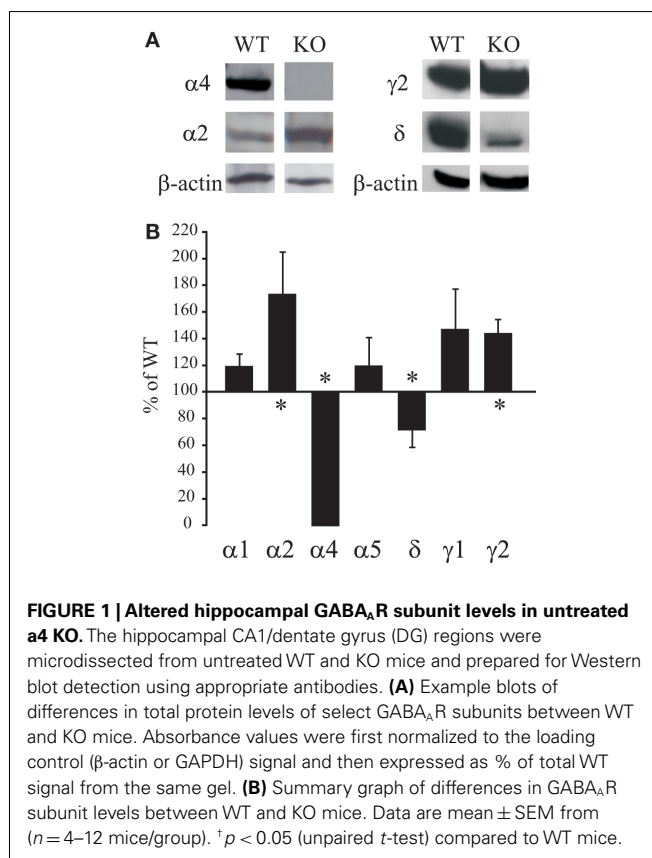


FIGURE 1 | Altered hippocampal GABA_AR subunit levels in untreated α 4 KO. The hippocampal CA1/dentate gyrus (DG) regions were microdissected from untreated WT and KO mice and prepared for Western blot detection using appropriate antibodies. **(A)** Example blots of differences in total protein levels of select GABA_AR subunits between WT and KO mice. Absorbance values were first normalized to the loading control (β -actin or GAPDH) signal and then expressed as % of total WT signal from the same gel. **(B)** Summary graph of differences in GABA_AR subunit levels between WT and KO mice. Data are mean \pm SEM from ($n = 4$ –12 mice/group). * $p < 0.05$ (unpaired t -test) compared to WT mice.

these were not observed, nor was there any significant change in α 1 subunit levels ($p = 0.25$).

ALTERED IDENTITY OF GABA_AR SUBUNITS INTERNALIZED BY EtOH TREATMENT IN α 4 KO MICE

We next compared the effects of *in vivo* EtOH exposure (3.5 g/kg, i.p.) on the intracellular levels of GABA_AR subunits between WT and KO mice. Since mice rapidly metabolize EtOH, all mice were pretreated with the alcohol dehydrogenase inhibitor, pyrazole (68 mg/kg, i.p.) 30 min prior to saline or EtOH treatment. Mice in both EtOH-treated groups maintained loss of righting reflex (LORR) by the time isoflurane anesthesia was administered (50–59 min post-EtOH). By comparing blots of microdissected slices incubated with or without the membrane-impermeable cross-linking reagent BS³, we were able to identify the intracellular pools of GABA_AR subunits (Grosshans et al., 2002). Cell-surface proteins form high molecular weight aggregates with BS³, such that they remain at the top of the gel. By contrast, intracellular proteins are not accessed by the membrane-impermeant reagent and thus can be quantified through Western blot analysis.

As illustrated in **Figure 2**, analyses of the intracellular levels of WT EtOH-treated, compared to saline-treated mice, revealed a significant increase in the internal levels of α 1 ($p = 0.02$), α 4 ($p < 0.01$), and δ ($p = 0.03$) subunits, whereas α 2, α 5, and γ 2 subunits remained unchanged ($p > 0.05$). These data are consistent with the rapid internalization of α 4/ δ subunit-containing GABA_ARs previously observed after EtOH intoxication in the

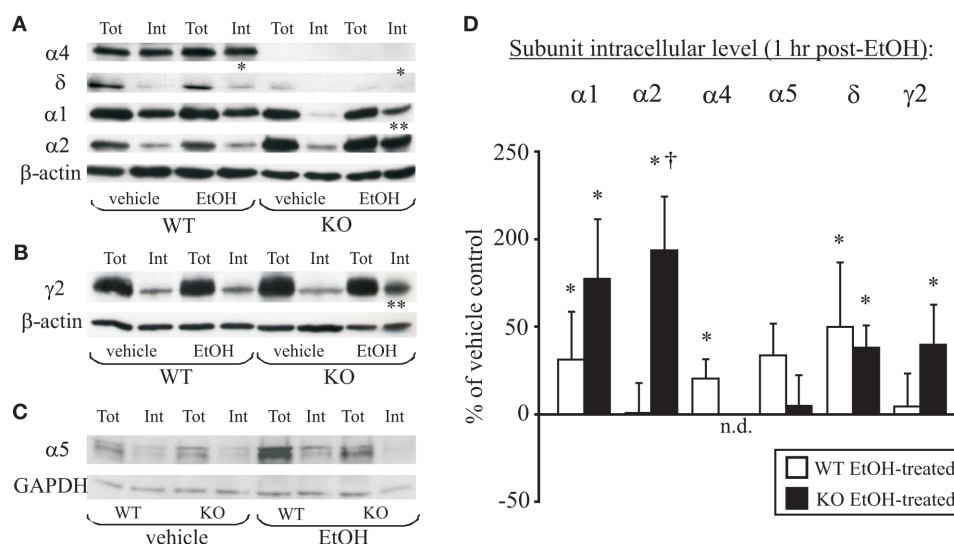


FIGURE 2 | EtOH-induced internalization of $\alpha 1$, $\alpha 2$, and δ subunits in the hippocampus of $\alpha 4$ KO mice. (A) Representative gels from the hippocampal CA1 and dentate gyrus (DG) regions microdissected from WT and KO mice at 1 h after vehicle (saline) or EtOH (3.5 g/kg, i.p.) injection. Note the increases in the intracellular fraction of $\alpha 1$, $\alpha 4$, and δ subunits (*) after EtOH treatment in WT mice. The $\alpha 4$ signal is absent while $\alpha 5$ and δ signal reduced in KO mice. In comparison to vehicle treatment, EtOH treatment induces internalization of $\alpha 1$, $\alpha 2$, δ and $\gamma 2$ subunits (*) in tissue collected from KO mice at 1 h after treatment. EtOH treatment also caused a significant increase in internalization of $\alpha 2$ subunits in KO mice (†) as compared to EtOH-treated WT mice. (B)

Similar experiments in a different gel illustrate internalization of the $\gamma 2$ subunit (*) at 1 h after EtOH treatment in KO but not WT mice. (C) Example gel illustrating the lack of $\alpha 5$ subunit internalization after EtOH treatment of WT or KO mice. (D) Summary graph of changes in internal subunit levels of WT (open bars) and KO (closed bars) mice 1 h after EtOH intoxication relative to vehicle-treated mice (horizontal 100% line). Data are mean \pm SEM ($n = 4$ –12 mice/group). The $\alpha 4$ signal was not detected (0%) in the $\alpha 4$ KO samples. * $p < 0.05$ (unpaired t -test) compares EtOH-treated with vehicle-treated mice; † $p < 0.05$ (unpaired t -test) compares WT EtOH-treated to $\alpha 4$ KO EtOH-treated groups.

rat (Liang et al., 2007). The EtOH-induced increases in internal $\alpha 1$ subunit levels are consistent with the presence of EtOH-sensitive $\alpha 1\beta\delta$ -GABA_ARs localized to hippocampal interneuron subpopulations in WT and $\alpha 4$ KO mice (Glykys et al., 2007). In EtOH-treated compared to saline-treated KO mice there was a significant increase in $\alpha 1$ ($p = 0.01$), $\alpha 2$ ($p = 0.01$), δ ($p = 0.02$), and $\gamma 2$ ($p = 0.046$) subunits, while $\alpha 5$ ($p > 0.05$) was unchanged and $\alpha 4$ subunit was not detected. The intracellular pool of $\alpha 2$ was significantly increased in KO EtOH-treated mice compared to WT EtOH-treated mice ($p = 0.02$). Consistent with our previous findings in rats (Liang et al., 2007), we also demonstrated a lack of EtOH-induced $\alpha 5$ subunit internalization in WT and KO mice (Figure 2C). These data suggested that in the absence of the highly EtOH-sensitive $\alpha 4/\delta$ subunit-containing GABA_ARs, EtOH intoxication results in the internalization of $\alpha 1$, $\alpha 2$, δ and $\gamma 2$ subunit-containing GABA_ARs in KO mice.

ALTERED ZOLPIDEM SENSITIVITY OF SYNAPTIC GABA_ARS IN $\alpha 4$ KO MICE

Based on the compensatory increases in $\alpha 2$ and $\gamma 2$ subunits in $\alpha 4$ KO mice (Figure 1), we hypothesized that synaptic GABA_ARs containing these subunits should also exhibit increased sensitivity to potentiation by benzodiazepine site ligands. To test this, we compared the responsiveness of mIPSCs in WT and KO mice to zolpidem (0.3 μ M). Zolpidem exhibits highest affinity for $\alpha 1\beta\gamma 2$ GABA_ARs; its affinity for $\alpha 2/3\beta\gamma 2$ GABA_ARs is 2–10-fold lower; its affinity for $\alpha 4/5$ -containing GABA_ARs is >1000-fold lower

(Wafford et al., 1993; Möhler et al., 2000). In hippocampal neurons of $\alpha 1$ KO mice, 0.3 μ M zolpidem still potentiates mIPSCs by prolonging their decay time (Goldstein et al., 2002). Thus, zolpidem at 0.3 μ M is expected to potentiate both $\alpha 1\beta\gamma 2$ and $\alpha 2\beta\gamma 2$ populations of GABA_ARs. We further hypothesized, based on the observed EtOH-induced internalization of $\alpha 1$, $\alpha 2$, and $\gamma 2$ subunit-containing GABA_ARs in $\alpha 4$ KO mice, that zolpidem potentiation of synaptic GABA_ARs should be selectively reduced by EtOH treatment in KO mice.

Analysis of mIPSC kinetics revealed differences between saline-treated WT and KO mice analogous to those reported in detail previously (Chandra et al., 2006; Liang et al., 2008). The main differences were decreased amplitude and frequency of mIPSCs in KO mice (Table 1). For both phenotypes, the mIPSC kinetics did not significantly differ between saline- and EtOH-treated mice, except for a post-EtOH increase in mIPSC decay τ_2 of WT mice.

In our recording conditions of 34.5°C the main effect of zolpidem was an increase in mIPSC decay time for both WT and KO DG cells (Table 2). As hypothesized, we observed significantly greater potentiation of mIPSCs by zolpidem in recordings from saline-treated KO mice compared to saline-treated WT mice (Table 2 and Figures 3A–C). Further analysis revealed that zolpidem potentiation of mIPSCs was unchanged at 1 h post-EtOH treatment in WT mice, but was significantly reduced at 1 h post-EtOH treatment in KO mice (Table 2 and Figures 3A–C). These data provided pharmacological evidence that the synaptic GABA_ARs in DGCs of $\alpha 4$ KO mice exhibit increased benzodiazepine sensitivity and

Table 1 | Kinetic properties of mIPSCs in DG cells from saline- and EtOH-exposed WT and KO mice.

	WT		KO	
	Post-saline	Post-EtOH	Post-saline	Post-EtOH
Amplitude (pA)	20.9 ± 1.0	19.0 ± 1.6	15.0 ± 1.8 [†]	15.8 ± 2.8
Rise time (ms)	0.7 ± 0.1	0.6 ± 0.1	1.4 ± 0.4	0.8 ± 0.2
Decay τ_1 (ms)	8.1 ± 0.4	7.7 ± 0.6	9.7 ± 2.2	8.5 ± 1.5
Decay τ_2 (ms)	12.3 ± 1.4	18.1 ± 1.9*	16.3 ± 2.7	18.3 ± 3.0
Area (fC)	291.3 ± 24.7	269.1 ± 21.1	263.9 ± 33.4	290.9 ± 45.7
Frequency (Hz)	12.3 ± 0.5	11.7 ± 0.5	10.2 ± 0.4 [†]	10.2 ± 1.2
n	9	15	6	5

* $p < 0.05$ from post-saline.

[†] $p < 0.05$ from similarly treated WT.

All data are expressed as % ± SEM.

Table 2 | Zolpidem (0.3 μ M) effects on mIPSC kinetics in DG cells from saline- and EtOH-exposed WT and KO mice.

	WT		KO	
	Post-saline	Post-EtOH	Post-saline	Post-EtOH
Amplitude	102.3 ± 0.8	100.8 ± 0.4	101.6 ± 7.5	106.3 ± 0.5
Rise time	126.2 ± 9.9	160.7 ± 16.4	168.6 ± 32.1	189.0 ± 41.4
Decay τ_1	156.9 ± 17.5	141.1 ± 11.8	279.9 ± 49.1 [†]	167.8 ± 10.6*
Decay τ_2	224.2 ± 85.9	216.1 ± 61.2	183.5 ± 14.5	156.0 ± 11.5
Area	142.4 ± 10.9	132.3 ± 8.1	213.4 ± 2.8 [†]	150.7 ± 4.0*
Frequency	100.7 ± 1.3	100.3 ± 2.3	99.7 ± 3.4	105.6 ± 1.1
n	6	5	3	4

* $p < 0.05$ from post-saline.

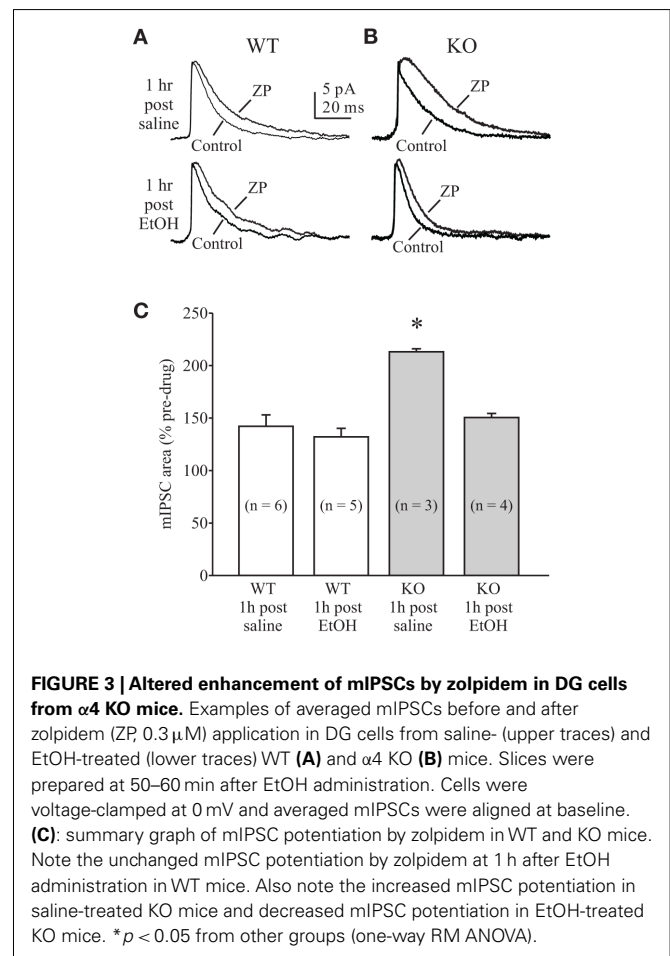
[†] $p < 0.05$ from WT.

All data are expressed as % ± SEM of a pre-drug parameter.

are rapidly internalized after exposure to high EtOH concentrations.

DISCUSSION

Here we demonstrate that global deletion of the $\alpha 4$ subunit produces compensatory alterations in the subunit composition and function of synaptic GABA_ARs. Specifically, loss of the $\alpha 4$ subunit leads to a reduction in δ subunit levels. Normally these subunits co-assemble into peri- and extra-synaptically located $\alpha 4\beta\delta$ -GABA_ARs (Nusser et al., 1998; Wei et al., 2003; Liang et al., 2006) which exhibit low conductance, high affinity for GABA (Wei et al., 2003; Chandra et al., 2006), insensitivity to BZs, and sensitivity to low millimolar concentrations of EtOH (Sundstrom-Poromaa et al., 2002; Wallner et al., 2003; Wei et al., 2004; Liang et al., 2008). Consistent with the extrasynaptic localization and functional properties of $\alpha 4\beta\delta$ -GABA_ARs, previous studies demonstrated that DGCs



of mice with global deletions of either δ or $\alpha 4$ subunits exhibit greatly reduced tonic inhibitory currents (Chandra et al., 2006; Liang et al., 2008) and reduced sensitivity to potentiation by acute EtOH (Glykys et al., 2007; Liang et al., 2008). Surprisingly, the synaptic currents in DGCs of δ KO (Liang et al., 2006), and particularly $\alpha 4$ KO (Liang et al., 2008) mice exhibit enhanced sensitivity to low concentrations of EtOH. This enhanced synaptic responsiveness provides a plausible physiological explanation for the apparently normal behavioral responses to acute EtOH of these KO mice, despite clear reductions in EtOH-enhanced tonic inhibitory currents in hippocampal neurons (Mihalek et al., 2001; Chandra et al., 2008). It must be noted that the normal responses to acute EtOH do not extend to chronic EtOH consumption or the more long-term effects of EtOH intoxication and subsequent withdrawal symptoms which are attenuated in δ KO mice.

Global loss of the δ subunit leads to compensatory decreases in $\alpha 4$ and increases in $\gamma 2$ subunit protein levels (Tretter et al., 2001; Peng et al., 2002). Increased co-immunoprecipitation of these remaining two subunits in δ KO mice (Korpi et al., 2002) suggests formation of functional synaptic GABA_ARs, which might be the cause of an enhanced responsiveness to low concentrations of EtOH (Liang et al., 2006). This conclusion is supported by the finding that rats withdrawn from acute or chronic EtOH administration exhibit marked increases in $\alpha 4$ and $\gamma 2$ subunit protein

levels, and increased sensitivity of synaptic GABA_ARs to low concentrations of acute EtOH (Liang et al., 2007). Chronic EtOH administration also results in large increases in $\alpha 4$ subunit incorporation within GABAergic synapses of DGCs (Liang et al., 2006). Here we demonstrate that global deletion of the $\alpha 4$ subunit leads to compensatory decreases in δ and increases in $\alpha 2$ and $\gamma 2$ subunit protein levels (**Figure 1**). These data confirm our previous findings of selective increases in $\gamma 2$ subunits in hippocampus and thalamus, but not cortex of $\alpha 4$ KO mice (Liang et al., 2008). Interestingly, we did not observe compensatory increases in the $\alpha 5$ subunit levels in the hippocampus. This subunit is normally expressed at relatively high levels in the extrasynaptic GABA_ARs of the CA1 region, which contribute to tonic inhibition (Caraiscos et al., 2004). Notably, $\alpha 5$ subunit-containing GABA_ARs also contribute to tonic inhibition in DG neurons because the reduced tonic currents in δ subunit KO are completely eliminated in the $\delta/\alpha 5$ double knockout mice (Glykys et al., 2008). The previously demonstrated large decreases in tonic currents of DG neurons from $\alpha 4$ KO mice (Liang et al., 2008) are consistent with the lack of compensatory increases in $\alpha 5$ subunit levels observed here. It would be interesting to determine if the magnitude of tonic currents in the CA1 region are affected by the loss of the $\alpha 4$ subunit.

An obvious question is whether the compensatory alterations in subunit composition are directly responsible for the altered function and pharmacological properties of synaptic GABA_ARs in $\alpha 4$ KO mice, particularly with respect to enhanced EtOH sensitivity (Liang et al., 2008). Previously, we localized a relatively small (~25%) but significant proportion of $\alpha 4$ subunits to the central portion of GABAergic synapses in DGCs of rats (Liang et al., 2006). Loss of such synaptic $\alpha 4$ subunits is consistent with the altered mIPSC kinetics of $\alpha 4$ KO mice (Chandra et al., 2006; Liang et al., 2008). Here we hypothesized that in the absence of $\alpha 4$ subunits, synaptic GABA_ARs with increases in relative proportions of $\alpha 2/\gamma$ subunits should also exhibit greater sensitivity to BZs. To test this, we compared synaptic responsiveness of WT and KO DGCs to zolpidem, a BZ site ligand which is inactive at $\alpha 4/5$ -containing GABA_ARs and preferentially potentiates $\alpha 1$ – $3/\gamma 2$ -containing GABA_ARs (Wafford et al., 1993; Möhler et al., 2000). Our data revealed significantly greater zolpidem potentiation of mIPSCs from KO mice compared to WT mice (**Figure 3**), supporting our hypothesis. However, the increased presence of $\alpha 2$ and $\gamma 2$ subunits at synapses does not explain the high EtOH sensitivity of these compensatory synaptic GABA_ARs. In addition to the multitude of novel subunit combinations, one cannot help suggest that some unknown factor(s) might also affect synaptic EtOH sensitivity. Perhaps this is related to GABA_AR-associated proteins and/or some protein phosphorylation event(s).

The presence of highly EtOH-sensitive synaptic receptors in tissues which virtually lack extrasynaptic tonic currents and EtOH responsiveness gives us the opportunity to explore several aspects of the EtOH-induced GABA_AR plasticity hypothesis (Liang et al., 2007). Here, we focused on the early events in EtOH intoxication, hypothesized to involve EtOH-induced overstimulation of EtOH-sensitive GABA_ARs resulting in their rapid internalization. Thus, we reasoned that if the EtOH-sensitive synaptic GABA_ARs of $\alpha 4$

KO mice were exposed to high doses of EtOH, we should be able to detect their internalization and its functional consequences with biochemical and electrophysiological techniques. Indeed, cross-linking and Western blot analysis of hippocampal tissue collected from KO mice within 1 h of EtOH exposure showed internalization of $\alpha 1$, $\alpha 2$, δ , and $\gamma 2$ subunits (**Figure 2**). By contrast, only $\alpha 1$, $\alpha 4$, and δ subunits showed EtOH-induced internalization in WT mice. Moreover, the zolpidem sensitivity of synaptic GABA_ARs of KO mice was significantly reduced at 1 h after EtOH treatment, whereas zolpidem sensitivity of WT mice was unchanged by EtOH treatment, suggesting functional decreases in $\alpha 1$ – $3/\gamma$ -containing synaptic GABA_ARs only in EtOH-treated KO mice.

Although δ subunit levels were significantly reduced in the $\alpha 4$ KO hippocampus, we could still detect EtOH-induced internalization of δ subunits in KO mice. Previous studies have demonstrated that unlike DGCs, certain hippocampal GABAergic interneurons express $\alpha 1/\delta$ -containing GABA_ARs which are responsive to low concentrations of EtOH (Glykys et al., 2007). We suspect that it is these remaining EtOH-sensitive δ -containing GABA_ARs that account for the EtOH-induced δ -subunit internalization signal in KO mice.

Use-dependent decrease of receptor function is a common mechanism of drug tolerance development. This process is a complex series of events which may include receptor desensitization, uncoupling between agonist binding and channel activation, receptor internalization, and transcriptional down-regulation. These separable mechanisms of drug tolerance development are specific to the class of receptors under study. For example, the development of acute tolerance to opioid analgesics was shown to involve receptor desensitization via phosphorylation and functional uncoupling of receptors from G proteins, while receptor internalization was shown to counteract desensitization through rapid recycling of opioid receptors back to the surface in a reactivated state (Koch and Holtt, 2008). Pathology-induced chronic opioid tolerance appears to involve transcriptional decreases in receptor expression (Abdulla and Smith, 1998). Acute tolerance to BZs is also thought to involve uncoupling between BZ and GABA binding sites of GABA_ARs (Hu and Ticku, 1994; Primus et al., 1996; Ali and Olsen, 2001). By contrast, development of acute tolerance to EtOH has been related to early internalization of extrasynaptic GABA_ARs (Liang et al., 2007), whereas protracted tolerance following chronic EtOH administration appears to involve transcriptional alterations in GABA_AR subunit composition and function (Matthews et al., 1998; Cagetti et al., 2003; Liang et al., 2006), reviewed in (Kumar et al., 2009). Fairly rapid transcriptionally mediated increases in $\alpha 4$ subunit levels have also been demonstrated following EtOH exposure (Pignataro et al., 2007). Such increases in $\alpha 4$ subunit levels were recently determined to require selective activation of the protein kinase γ isozyme (Werner et al., 2010). Rapid decreases in inhibition and BZ tolerance during status epilepticus have also been demonstrated to involve internalization of GABA_ARs (Goodkin et al., 2005, 2008; Naylor et al., 2005) and subsequent long-term alterations in GABA_AR subunit composition were demonstrated to involve transcriptional events (Brooks-Kayal et al., 1998; Roberts et al., 2005).

In summary, our studies showed that global genetic deletion of the $\alpha 4$ subunit leads to compensatory changes in synaptic GABA_AR subunit composition and function. These functional changes include increased zolpidem sensitivity. We also demonstrate that EtOH intoxication in the KO mice leads to rapid internalization of these zolpidem-sensitive synaptic

receptors. The $\alpha 4$ KO mice should prove useful in examining the long-term consequences of EtOH withdrawal and dependence.

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